Journal of Surgical Oncology 63:87–90 (1996)

p53 Gene Mutation Is Not Directly Related to Tumoricidal Effects of Preoperative Radiochemohyperthermia Therapy for Rectal Cancers

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Background: Several studies have recently demonstrated that apoptosis of cancer cells is triggered by diverse adjuvant cancer therapies and the induction of apoptosis correlates with the sensitivity of the primary tumor to such therapies.

Methods: We investigated the factors modulating adjuvant cancer therapies by examining p53 gene mutations and chromosome 17p allelic losses in 15 rectal cancers treated by a preoperative combined therapy consisting of radiation, intraluminal hyperthermia and 5-fluorouracil suppositories.

Results: The point mutations were detected in 7 of 15 (46.7%) tumors by single-stranded conformational polymorphism and direct sequencing. Allelic losses at chromosome 17p were also detected in 7 of 15 (46.7%) tumors by dinucleotide-repeat polymorphisms. There was no correlation between p53 gene abnormalities and the preoperative tumoricidal effect of the therapy.

Conclusions: We conclude that p53 gene abnormalities do not directly increase resistance to the combined adjuvant therapy.

KEY WORDS: p53, cancer therapy, sensitivity, apoptosis

INTRODUCTION

Local recurrence is the most predominant prognostic factor for patients with advanced rectal cancer, and various adjuvant therapies have been attempted to prevent it [1]. Among these adjuvant therapies, the most powerful is undoubtedly high-dose radiation or radiation combined with other therapies [2]. We developed a novel preoperative therapy combining radiation, intraluminal hyperthermia, and 5-fluorouracil suppositories for advanced rectal cancer [3]. This therapy led to a striking tumoricidal effect and resulted in improved prognosis for patients with advanced rectal cancers [3,4]. Our therapeutic protocol, as well as those reported by other investigators, showed varying degrees of tumoricidal effects among primary tumors [4], but little is known about the mechanism underlying the difference.

Apoptosis is a genetically encoded cell death program defined by characteristic morphologic and biochemical changes [5]. In recent years, several studies have demonstrated that apoptosis is triggered by diverse cancer therapies, and its degree correlates with sensitivity to cancer therapies [6–9]. The p53 tumor suppressor gene is recognized as one of the most important modulators of apoptosis [10,11]. Although several previous studies have attempted to clarify the relationship, it remains controversial whether p53 status correlates with the sensitivity to cancer therapy or not [12–16].

In this study, we examined the relationship between p53 gene status and sensitivity to radiochemohyperthermia therapy.

Accepted for publication May 28, 1996.

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MATERIALS AND METHODS

Patients and Tissue Samples

Thirty-six patients with advanced cancers (T3NxM0 and T4NxM0) in the lower rectum received a preoperative combination therapy of radiation, hyperthermia, and 5-fluorouracil (5-FU) suppositories prior to surgery [3,4]. The protocol was as follows: (1) a total irradiation dose of 30 Gy in 10 fractions every 2 days, (2) five applications of intraluminal hyperthermia (at 43–45°C for 50 min) within 1 hour after irradiation, and (3) 5-FU suppository (equivalent to 100 mg of 5-FU) every day for 20 days. Patients underwent surgery 1 week after completion of the combination therapy.

We examined 15 of these patients whose biopsy samples prior to the therapy were available. Surgically resected specimens were subjected to histological evaluation and classified into four categories: no remarkable changes (Grade 0); swelling of cells, enlarged vesicular nuclei, pycnosis, and vacuolated cytoplasm occupying <25% of tumor regions (Grade 1); cell nests consisting of markedly damaged cells, often exhibiting a moth-eaten appearance and simplified granular structures (Grade 2); extensive degenerative changes and fibrosis (>75%) (Grade 3) [4]. The histological grading of surgically resected specimens was compared with the genetic evaluation of the biopsy samples obtained prior to the preoperative therapy.

The endoscopic tumor samples and adjacent normal tissues were subjected to DNA extraction, performed as described previously [17]. The samples in which cancer cells predominated were used for the molecular studies noted below.

Polymerase Chain Reaction-Single-Stranded Conformational Polymorphism (PCR-SSCP) Analysis and Direct Genomic Sequencing

PCR-SSCP of the p53 gene was performed as described earlier [18]. PCR was performed with 100 ng of genomic DNA after 1 μCi of [α-^{32}P]deoxycytidine 5′-triphosphate was added to each reaction mixture. The sequences of the oligonucleotide primers were 5′-CTCTTCCTGCAG-TACTCCCCTGC-3′/5′-GCCCCAGCTGCTCACCATC-GCTA-3′ for exon 5, 5′-ACGACAGGGCTGGTTG-CCCA-3′/5′-CTCCCAGAGACCCCAGTTGC-3′ for exon 6, 5′-GGCCTCATCTCGGGCCTGTG-3′/5′-CAGTGT-GCAGGGTGGCAAGT-3′ for exon 7, 5′-CTGCCT-CTTGCTTCTCTTTT-3′/5′-TCTCCTCCACCGCTTCT-TGT-3′ for exon 8 and 5′-GCCTCTTTCCTAGCACTG-CCCAAC-3′/5′-CCCAAGACTTAGTACCTGAAGGG-TG-3′ for exon 9. The PCR products were diluted, denatured, and electrophoresed on 6% polyacrylamide gel containing 5% glycerol. The gel was then dried and exposed to X-ray film for 48–72 hours at −80°C.

To confirm the mutation, every PCR product showing mobility shift by SSCP analysis was directly sequenced by the dideoxy termination method using end-labeled sequencing primers and the T7 Sequencing Kit (Pharmacia LKB). The reaction was followed by electrophoresis on 8% polyacrylamide gel containing 7 mol/L urea.

TABLE I. p53 Gene Mutations and Chromosome 17 Allelic Losses in Rectal Cancer Underwent Radiochemohyperthermia Therapy

Case	Grade	Codon	Mutation	# Amino acid[a]	No. of 17p allele
1	1		ND		2
2	1		ND		2
3	2		ND		2
4	2		ND		2
5	2		ND		1
6	3		ND		2
7	3		ND		2
8	3		ND		2
9	1	196	1bp deletion	Stop at 246	1
10	1	159	GCC→GTC	Ala→Val	2
11	2	175	CGC→CAC	Arg→His	1
12	2	248	CGG→TGG	Arg→Trp	1
13	2	249	AGG→AAG	Arg→Lys	1
14	3	166	TCA→TAA	Ser→Stop	1
15	3	275	1bp insertion	Stop at 305	1

[a] Ala = alanine; Val = valine; Arg = arginine, His = histidine, Trp = tryptophan; Lys = lysine; Ser = serine.
ND = not detected.

Allelic Deletion Analysis

To measure allelic deletions, PCR-loss of heterozygosity (LOH) analysis using a microsatellite marker (TP53 [19]) within the p53 gene was performed as previously described [20]. In a few cases in which the probe was not informative, additional markers [21] at chromosome 17p13 were analyzed.

RESULTS

According to the tumoricidal effect detected in the resected specimens, there were four Grade 1 tumors, six Grade 2 tumors, and five Grade 3 tumors.

SSCP analysis showed abnormally migrating in seven (46.7%) of the 15 tumors; three in exon 5, one in exon 6, two in exon 7, and one in exon 8 (Fig. 1A; Table I). All of the abnormalities were identified by direct sequencing and are listed in Table 1. Five of these tumors had missense mutations, and the remaining two cases had nonsense mutations. Four of the five point mutations were transitions; cases 11, 12, and 13 contained point mutations at codons 175, 248, or 249 known as "hot spot" codons (Fig. 1B). In cases 9 and 15, deletion and insertion would be expected to produce premature stop codons.

On the other hand, chromosome 17p allelic loss was also detected in 7 (46.7%) of 15 tumors by dinucleotide-repeat polymorphisms (Fig. 2; Table I). Six of these seven tumors also contained p53 gene mutations, but in the

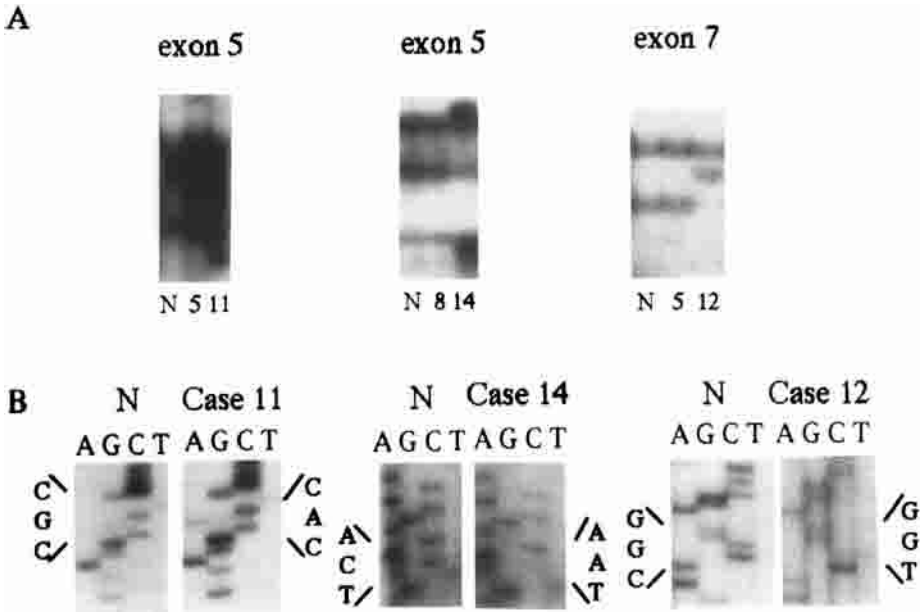

Fig. 1. **A.** PCR-SSCP analysis of the p53 gene. The case number is shown at the bottom of each lane. Lane N is control DNA. Cases 11 and 14 (exon 5) and 12 (exon 7) show different mobilities. (PCR-SSCP = polymerase chain reaction - single stranded conformational polymorphism) **B.** Direct genomic sequence analysis of the p53 gene in case shown in Fig. 1A. Mutated sequences were compared with a control (DNACN) containing normal sequence of p53. Case 11, at codon 175 (G → A) in exons; Case 14, at codon 166 (C → A) in exons; Case 12, at codon 248 (C → T) in exon 7.

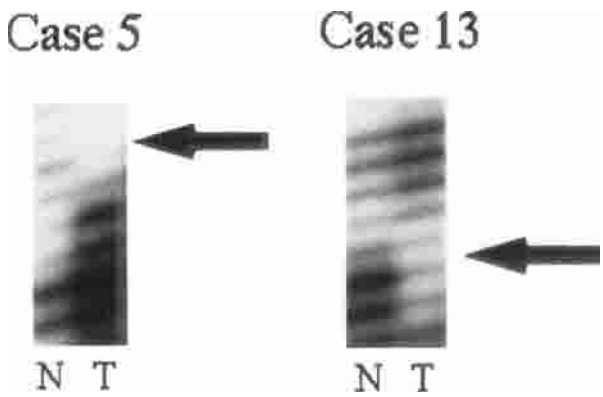

Fig. 2. PCR-LOH analysis of a CA repeats at the p53. T : Tumor DNA, N : normal (constitutional) DNA. Arrows indicate allelic deletions in each case. (PCR-LOH = polymerase chain reaction - loss of heterozygocity)

remaining one case with allelic loss, p53 gene mutation in exon 5-9 was not found by this assay.

Table II summarizes the findings according to the degree of therapeutic effect. p53 gene abnormalities were detected in two of the four (50.0%) Grade 1 tumors (resistant tumor), four of the six (66.7%) Grade 2 tumors (intermediate tumor), and two of the five (40.0%) Grade 3 tumors (sensitive tumor). Of these abnormalities, p53 point mutations were detected in two Grade 1 tumors (50.0%), three Grade 2 tumors (50.0%), and two Grade 3 tumors (40.0%). The point mutation also correlated well with allelic loss at chromosome 17p in this study, in agreement with other investigators [22,23].

TABLE II. Correlation between p53 Gene Abnormalities and Therapeutic Effect of Combined Therapy

Grade[a]	Wild/wild	Mutant or allelic loss	Total
1	2	2	4
2	2	4	6
3	3	2	5

[a]Grade 1 = resistant tumor; grade 2 = intermediate tumor; grade 3 = sensitive tumor.

DISCUSSION

For some time there has been considerable interest in the sensitivity to cancer therapy, but little is known about the cause of the variation in the response of cancers to adjuvant cancer therapy. One hypothesis is that the sensitivity might depend on the dividing speed of the cancer cells. However, this explanation is not satisfactory, because the majority of rapidly dividing cancers are resistant to therapy in the clinics.

Recently, induction of apoptosis in cancer cells has been demonstrated to correlate well with the sensitivity to cancer therapy and to be modulated by a multitude of factors, including growth factors, intracellular mediators of transduction, and nuclear proteins regulating gene expression, DNA

replication, and cell cycle [24]. Among them, the p53 tumor suppressor gene is recognized as one of the most important modulator of apoptosis [10,11]. In normal cells, wild-type p53 gene product plays an important role in the apoptosis pathway and cell cycle arrest after DNA damage [10]. Therefore, p53 gene mutations are supposed to lack these functions through normal pathways in tumor cells.

The relationship between the p53 gene and apoptosis or sensitivity to cancer therapy has been investigated [12–16]. Lowe et al. [14] have demonstrated using p53-deficient mouse embryonic fibroblast that p53 null murine cell did not respond to radiation and anticancer drugs. They also demonstrated that a few point mutations in p53, inactivating the gene, could produce treatment-resistant tumors and then suggested that p53 status is an important determinant of tumor response to therapy [16]. Some other investigators have also demonstrated similar results using human lymphoma cell lines [13]. In contrast, Strasser, et al. [15] showed, using $p53^{-/-}$ mice, that activated lymphocytes and T-lymphoma cells from $p53^{-/-}$ mice underwent apoptosis after irradiation or genotoxic drug treatment and concluded that p53 gene mutation did not affect the responsiveness of cancer cells to adjuvant cancer therapy, and consequently p53 is not the only mediator of apoptosis provoked by DNA damage. Using 24 head and neck cancer cell lines, the other group also demonstrated a similar conclusion that cell killing following irradiation is not decreased by p53 mutations [12].

In the present study, we found no correlation between p53 gene abnormalities (mutation frequency, mutation location, type of mutation, allelic loss at chromosome 17p) and cell killing after DNA damage in rectal cancers. Since hyperthermia has been known to enhance cell killing through apoptosis in connection with normal p53 protein [8,25], our findings may support that the tumoricidal effect following radiochemotherapy is not decreased by p53 mutations and indicates that wild-type p53 is not just a trigger for inducing a tumoricidal effect following radiochemohyperthermia therapy. Strasser et al. [15] advocated the simplest model to apoptosis. Their model has p53-dependent and p53-independent multiple routes but a final common pathway to apoptosis after DNA damages, and Bcl-2 can inhibit these several routes to apoptosis. The inhibition of the apoptosis pathway might have some clues to analysis for the responsiveness of cancer cells to adjuvant cancer therapies.

In conclusion, our findings suggest that p53 mutations do not directly increase resistance to powerful radiochemohyperthermia combined therapy.

ACKNOWLEDGMENTS

We thank Dr. Shinichi Misawa, Dr. Shigeo Horiike, and Dr. Hiroto Kaneko, Third Department of Medicine, Kyoto Prefectural University of Medicine, for their technical advice.

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